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Consistent Polymerse Chain Reaction–Single-Strand Conformation Polymorphism Pattern of Human Herpesvirus-8 in the Course of Classical Kaposi's Sarcoma Assumes Its Clonal Origin

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There is emerging evidence that Kaposi's sarcoma–associated herpesvirus (KSHV or HHV-8) has a central role in the pathogenesis of Kaposi's sarcoma (KS). The occurrence of HHV-8 in classical KS biopsies is reported irrespective of its clinical stage (patch, plaque, nodular). HHV-8 was detected in 25 of 28 formalin-fixed paraffin-embedded classical KS samples by nested polymerase chain reaction. In addition, in six patients multiple tumors were available (n = 21). Single-strand conformation polymorphism (SSCP) analysis of the amplicons showed uniform SSCP pattern of samples belonging to the same patient regardless of whether the KS was multiplex or developed again years after the first excision. Most of the SSCP patterns were confirmed by further sequence analysis. The presence of the same sequence variant of HHV-8 in various samples of the same patient supports the clonal origin of classical Kaposi's sarcoma. ***J. Med. Virol. 54:300–304, 1998.***

KEY WORDS: human herpesvirus-8; Kaposi's sarcoma; clonal neoplasm

INTRODUCTION

Kaposi's sarcoma (KS) was described originally in 1872 by Mor Kaposi. The classical form of Kaposi's sarcoma (CKS) is known as a vascular neoplastic lesion predominantly of the lower extremities in elderly men with often Jewish or Mediterranean origin [Zalla, 1996; Tappero et al., 1993]. The other forms of KS such as African endemic KS, AIDS-associated KS (AKS), and iatrogenic post-transplant KS have aggressive clinical phenotypes with often multicentric, generalized appearance. Atypical neoangiogenesis, spindle cells, and inflammatory cell infiltration are histological characteristics of KS [Tappero et al., 1993]. The spindle cells are thought to be the neoplastic component of the lesion. Several lesions in different stages (patch, plaque, tumor) can occur in the same time and may regress or develop further independently from each other [Zalla, 1996]. On the basis of the clinical behavior it is unclear whether KS is a true neoplasm or a multicentric reactive hyperplasia [Costa, 1983; Brooks, 1986]. Reactive proliferative origin rather than a clonal neoplasm was supported by flow cytometric DNA analysis [Fukanaga et al., 1989], by Feulgen-based DNA analysis, and by cyclin immunochemistry [Kaaya et al., 1992]. Polyclonality was shown by different chromosomal abnormalities of primary AKS cultures [Bovi et al., 1986]. Recently clonality of spindle cells was suggested by the monoclonal pattern of inactivation of X chromosomes [Rabkin et al., 1995] and by the uniform methylation pattern of androgen-receptor gene in AKS [Rabkin et al., 1997].

Before and after the onset of the AIDS epidemics several studies suggested an infectious pathogen as the causative agent for KS [Beral et al., 1992]. The high (90–100%) prevalence of recently identified herpesvirus-like DNA sequences [Chang et al., 1994] belonging to the new γ-herpesvirus, human herpesvirus-8 (HHV-8) have been confirmed by many investigators in all forms of KS from different geographical regions [Huang et al., 1995; Rady et al., 1995a]. HHV-8 presents in the spindle cells and in atypical endothelial cells of the KS lesion [Boshoff et al., 1995]. The prevalence of antibodies to HHV-8 latent nuclear antigens is

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significantly higher in KS patients and in KS risk groups than in healthy blood donors [Kedes et al., 1996]. In addition, seroconversion [Gao et al., 1996] and detection of HHV-8 DNA in PBMCs of HIV+ patients preceded the KS development [Whitby et al., 1995]. Genetic homology data indicate that HHV-8 has open reading frames that can play an active role in the pathogenesis of KS [Cesarman et al., 1996; Russo et al., 1996]. At present, HHV-8 is the candidate agent for KS, but some studies reported detectable HHV-8 also in non-KS lesions. These unconfirmed results question the specific role of HHV-8 in KS [Rady et al., 1995b; Gyulai et al., 1996; McDonagh et al., 1996]. However, the detection of HHV-8 DNA in these samples could be accidental since HHV-8 is a widespread virus in adult populations indicated by 25% prevalence of anti-lytic antibodies in U.S. blood donors [Lenette et al., 1996]. Detectable HHV-8 DNA in semen and mRNA in prostatic tissue samples correspond well with possible sexual transmission [Monini et al., 1996; Staskus et al., 1997].

We now provide additional data confirming the prevalence of HHV-8 in CKS tissue samples irrespective of its clinical stage. Further polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) and sequencing analysis of multiple lesions from several patients suggest the clonal origin of CKS and the possible pathogenic role of HHV-8 in KS.

PATIENTS AND METHODS

Patients

Twenty-eight CKS involved in the PCR studies belonged to 13 patients. All of the lesions were excised in toto from patients in the Department of Dermatology, University Medical School of Debrecen in the period of 1991 to 1996. Prior to histological examination, tissues were fixed in buffered-formalin for 4 to 8 hr, embedded in paraffin, and stained by the standard hematoxylin and eosin method. In the case of six patients, multiple tumors were available (n = 21). Samples of four patients were excised from different locations at different time points. The time between surgical treatment ranged from a few months to several years. Samples of two patients were excised from different locations at the same time. Non-KS angiomatous tissues such as pyogenic granulomas (2), hemangiomas (2), and non-angiomatous tumor melanoma (2) and basalioma (2) were the negative controls.

Methods

DNA preparation. Three sections (20 μm thick each) were deparaffinized by 2 × 1 ml xylene for 5 min each. After washing with 2 × 1 ml ethanol for 2 min samples were digested with 400 μl 250 μg/ml Proteinase-K in 1 × PCR buffer (10 × PCR buffer: 500 mM KCl, 100 mM Tris-HCl pH = 9, 1% Triton X-100) for 15 min at 65°C. Between the steps samples were spun down at 10,000*g* for 5 min. After digestion, the supernatant was discarded and the pellet was hydrolyzed in 200 μl 0.1 N NaOH for 10 min at 95°C. Samples were neutralized by the addition of 100 μl 1 M Tris-HCl pH = 1. The neutralized centrifuged hydrolysates (approx. 250–300 μl) were added to 1 ml 96% ethanol, and DNA was precipitated for 1 hr at −20°C. Pellet was dissolved in 50 μl TE (10 mM Tris-HCl pH = 8, 0.1 mM EDTA) after centrifugation for 30 min at 13,000*g,* 4°C.

PCR amplification. Five microliters of dissolved DNA was used for PCR studies. DNA quality control was carried out by amplification of the β-globin gene with the primers PCO3 (aca,caa,ctg,tgt,tca,cta,gc) and PCO4 (caa,ctt,cat,cca,cgt,tca,cc) (1.5 mM $MgCl_2$, 30 cycles with 95–55–72°C for 1 min each). HHV-8 sequences have been amplified with nested-PCR, the second round serving as specificity control and increasing sensitivity. The amplified sequences represent the putative minor capsid protein of HHV-8; outer primers [described by Chang et al., 1994] and inner primers [by Whitby et al., 1995] generate 233 and 172 bp length amplicons, respectively. One microliter after the first 20 cycles served as target for additional 25 cycles in the nested round. For the PCRs 50 pmole of each primer, 1 unit Taq polymerase, 2 mM $MgCl_2$ in 1 × PCR-buffer were used in a final volume of 25 μl. Cycles were carried out at 95–58–72°C for 1 min each. Carryover contamination was avoided by using filtered pipet tips and opening tubes singly after centrifugation. All samples were examined twice including independent DNA preparations and PCR amplifications. The independent tests gave equal results. No sample-to-sample contamination occurred since the non-KS and -DNA/H_2O controls were negative for target sequences. In our hands this protocol gave positive signal from 0.7 pg DNA from a native CKS as positive control. Fifteen microliters of the nested PCR products was electrophoresed in 1.5% agarose gel stained with ethidium-bromide, visualized, and photographed by UV transillumination (Fig. 1).

SSCP analysis. Three microliters of each nested PCR product was diluted with 100 μl formamide (0.05% xylene cyanol and bromphenol blue). Twenty microliters of each diluted sample was electrophoresed in 1 × TBE/10% polyacrylamide gel at 7°C, 200 V. Single-stranded DNA was visualized by silver staining (Fig. 2).

Sequencing. Cycle sequencing was undertaken on one strand of the nested PCR products with Promega *f*mol DNA Cycle Sequencing System according to the manufacturer's recommendations in two separate runs.

RESULTS

Twenty-five of 28 CKS samples were positive for HHV-8 by nested PCR (Table 1). HHV-8 was not detected in non-KS angiomatous tissues such as in benign angiomas, pyogenic granulomas, and in non-angiomatous tumors, namely melanoma and basalioma. HHV-8 was detected at all stages of KS, but at lower frequencies in early stage lesions (Table I). The small number of KS cells in these sections may lead to the lower rate of HHV-8 detection. The high prevalence of HHV-8 in KS versus non-KS tissues and the distri-

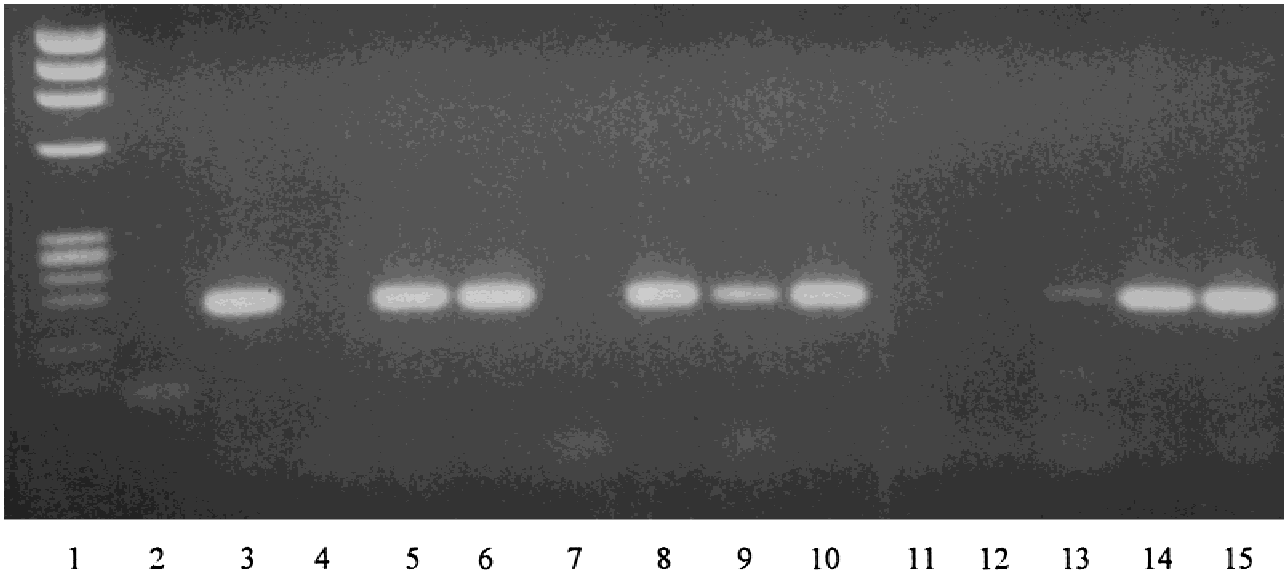

Fig. 1. Nested PCR amplification 172 bps of HHV-8. **Lane 1:** ΦX174 Hae III; **lanes 2,4:** –DNA/H_2O controls; **lane 3:** native CKS/+ control; **lanes 5–15:** formalin-fixed/paraffin-embedded tissues; **lanes 5–10, 13–15:** CKS samples; **lane 11:** pyogenic grnauloma; **lane 12:** hemangioma.

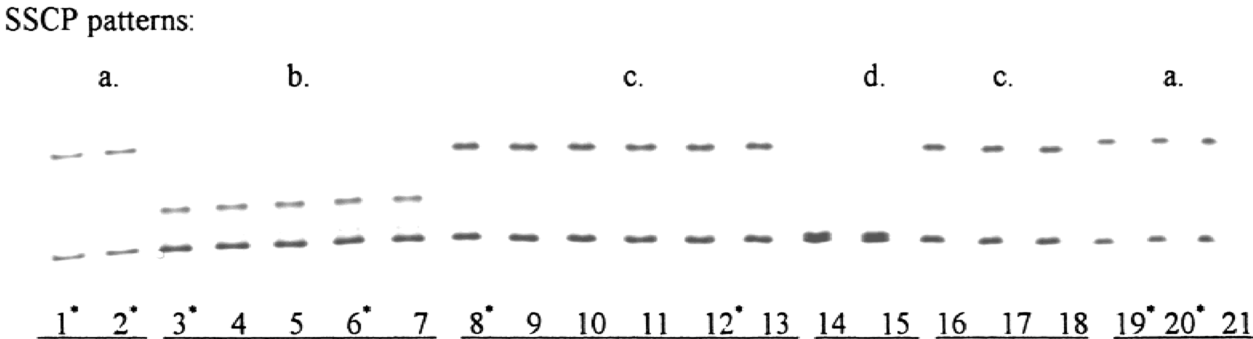

Fig. 2. PCR-SSCP patterns of multiple samples of six patients (sampling time). Well-distinguishable mobility shifts can be seen. Only the patterns in lanes 1, 2 and 19–21 were indistinguishable from each other; however, they represented different sequence variants. Samples in lanes belonging to the same patient are underlined separately. Patient 1: **lanes 1, 2** ('91, '94); patient 2: **lanes 3–7** ('92, '93, '94, '94, '95); patient 3: **lanes 8–13** ('92, '94, '94, '95, '95, '96); patient 4: **lanes 14, 15** ('94 all); patient 5: **lanes 16–18** ('94 all); patient 6: **lanes 20, 21** ('94 all). *, Samples were sequenced.

bution of HHV-8 in all stages of KS support its possible pathogenic role in KS as suggested by others.

Several genetic variants as point mutations are often within this region as shown by other results [e.g., Rady et al., 1995a; Huang et al., 1995]. Sequence analysis of the amplified products was carried out in case of multiple (total of 21) samples of six patients. SSCP analysis of PCR products revealed four distinguishable different SSCP patterns (a, b, c, d) representing different point mutations in the amplified region (Fig. 2). Sequencing analysis revealed the sensitivity and specificity of the SSCP analysis. Sequencing of three different SSCP patterns (a, b, c) revealed four different sequence variants (Table II). Samples belonging to the same patient regardless of the sampling time or the location of multiplex lesions showed uniform SSCP patterns (Fig. 2) and sequence variants (Table II). Due to the uniform SSCP patterns of different KS samples of the same patient, the lesions should contain the same sequence variant of HHV-8.

TABLE I. Sample Stage and HHV-8 Detection in Classical Kaposi's Sarcoma Samples by Nested PCR

Stage	HHV-8+	HHV-8−	Total
Patch	1	2	3
Plaque	5	1	6
Nodular	19	0	19
Total	25	3	28

DISCUSSION

The clonal or polyclonal origin of KS is still controversial [e.g., Bovi et al., 1986; Rabkin et al., 1995; Rabkin et al., 1997]. The analysis of cellular genomic DNA content of KS lesions may lead to contradictory

TABLE II. Point Mutations in the Sequenced Region Compared to the Published Sequence [Chang et al., 1994]

SSCP pattern	Nucleotide changes	Lane No. in Fig. 2
a	1033. C > T 1146. G > A	1, 2
b	1032. C > A 1033. C > T 1132. A > G 1139. A > C	3, 6
c	1033. C > T	8, 12
a	1033. C > T 1145. G > A	19, 20

results even with the same method [Rabkin et al., 1997; Delabesse et al., 1997] since lesions contain various cell populations [Tappero et al., 1993]. The spindle cells of KS lesions harbor the HHV-8 genome in latent circular form [Decker et al., 1996]. The consequences of viral latency are the minimal level of replication and infrequent mutations, so HHV-8 DNA analysis would be suitable for judging the clonality of KS. The consistent SSCP patterns correspond well with the latent viral phase and suggest the clonal origin of different lesions from the same patient. In the case of polyclonal origin, several different sequence variants represented by different SSCP patterns would be detected.

In addition, the peripheral blood mononuclear cells (PBMCs) of KS patients are infected lytically by HHV-8 [Decker et al., 1996]. During the lytic phase in the PBMCs new point mutations occur in the HHV-8 genome compared to that of the lesions [Decker et al., 1996]. It is not probable that the same sequence variant of HHV-8 from lytically infected PBMCs infects secondary several lesions independent from each other in place or time.

In summary, the latent HHV-8 infection of KS lesions is restricted to spindle cells, so the consistent SSCP patterns of HHV-8 indicate the clonal origin of different KS lesions, which corresponds well with the findings of Rabkin et al. [1997]. Our result also supports the theory of the pathogenic role of HHV-8 in KS. Neoplastic spindle cell clone could emerge from HHV-8–infected cells of yet undetermined origin. The possible transforming event would involve the effects of viral *cyclin D-1* and viral *G-protein coupled receptor* coding regions on cell proliferation as observed in transfection experiments [Chang et al., 1996; Arvanitakis et al., 1997].

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